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(54) Title: METHODS TO SCREEN MICROORGANISMS OR GENE LIBRARIES FOR PRODUCTS SECRETED FROM A CELL

(57) Abstract: The invention describes methods for screening for products secreted from the cells, and provides methods to establish a correlation between the activity of the secreted product and the secreting cell. Accordingly in a first aspect the present invention relates to a method for screening a DNA library for DNA of interest comprising the steps of a) creating host cells comprising the DNA library, b) generating samples each comprising a host cell of step a), c) establishing a means for correlating host cell secretion of a material of interest in a sample with the fluorescence of the sample, d) determining which intensity interval of fluorescence indicates secretion in the sample when the correlating means of step c) is used, e) cultivating the samples under suitable conditions, and f) selecting the samples exhibiting fluorescence within the intensity interval of step d) using a fluorescence analyzer; wherein the host cell comprises DNA of interest.

Methods to screen microorganisms or gene libraries for products secreted from a cell.

Field of invention

Microbial organisms are widely used for the manufacture of various industrial products and optimizing the production in microbial hosts with respect to yield and production cost has become a highly competitive area of research. The microbial production of such products as industrial enzymes or pharmaceuticals is usually achieved by a fermentation process followed by one or more purification steps.

Products that are secreted from the producing microorganism into the fermentation broth are particularly preferred in the industry, since secretion minimizes the required post-fermentative purification. Methods to efficiently screen microorganisms or gene libraries for production of secreted products have consequently become of great importance.

Background of the invention

Use of a Fluorescence Activated Cell Sorter (FACS) to screen for enzymes is described in the art.

WO 98/58085 describes screening gel microdrops (GMD's) for an intracellular enzyme (β-galactosidase).

Nir et al. (Nir, R., Y. Yisraeli, R. Lamed, and E. Sahar. 1990. Flow cytometry sorting of viable bacteria and yeasts according to beta-galactosidase activity. Appl. Environ. Microbiol. 56:3861-3866) developed a method for sorting cells on the basis of expression of β-galactosidase using a fluorescent substrate. The β-galactosidase assay required partial permeabilization of cells and 'cross-talk' (i.e. exchange of metabolites) between GMD's was minimized by carrying out the β-galactosidase assay at 4°C.

Intracellular esterase activity has been measured in *Pseudomonas aeruginosa* using 6-carboxy-fluorescein-diacetat,

and an assay measuring the disappearance of fluorescence from GMD's containing fluorescently labeled casein was described for elastase secreted by *Pseudomonas aeruginosa* (Sahar, E., R. Nir, and R. Lamed. 1994. Flow cytometric analysis of entire microbial colonies. *Cytometry* 15:213-221).

Cid et al (V. J. Cid, A. M. Alvarez, A. I. Santos, C. Nombela, and M. Sanchez. 1994. Yeast exo-beta-glucanases can be used as efficient and readily detectable reporter genes in *Saccharomyces cerevisiae*. *Yeast* 10 (6): 747-756) describe a FACS based reporter system using a β-glucanase that partially accumulates in the yeast periplasm.

WO 99/10539 describes an assay for bioactive substances by co-encapsulation of library and target cells and sorting on the basis of e.g. live/dead staining. A similar approach using co-encapsulation of cells in a screening for natural compounds was described in WO 98/41869.

WO 98/49286 discloses a surface display system where a protease (OmpT) is retained on the cell surface and a substrate is modified to bind to the cell by electrostatic interactions. Protease action is detected by a FACS via de-quenching of a Bodipy-FL tri-methyl-rhodamine amino acid substrate where the BIDOPY-FL part is retained at the cell surface.

In US 4,401,755 coating GMD's is indicated as a way to make GMD's essentially impermeable to the substrates or products of the activity screened for.

Summary of the invention

The present invention provides a solution to the problem of how to screen for products secreted from microbial cells.

Screening for e.g. secreted enzymes that degrade substrates which are not taken up by the cells requires overcoming several difficulties, the secreted enzymes diffuse from the cells they originate from, and the extracellularly degraded substrate molecules may diffuse freely between cells or GMD's;

thus the correlation between secreting cells and degraded substrate is usually lost.

The invention describes a method for screening for products secreted from cells, and provides means to establish a correlation between the activity of the secreted product and the secreting cell.

Accordingly in a first aspect the present invention relates to a method for screening a DNA library for DNA of interest comprising the steps of a) creating host cells comprising the DNA library, b) generating samples each comprising a host cell of step a), c) establishing a means for correlating host cell secretion of a material of interest in a sample with the fluorescence of the sample, d) determining which intensity interval of fluorescence indicates secretion in the sample when the correlating means of step c) is used, e) cultivating the samples under suitable conditions, and f) selecting the samples exhibiting fluorescence within the intensity interval of step d) using a fluorescence analyzer; wherein the host cell comprises DNA of interest.

There are a number of standard ways known in the art to express a DNA sequence of interest once such a sequence has been isolated (see below).

Consequently in a second aspect the present invention relates to a recombinant vector comprising DNA isolated by a method as defined in the first aspect of the invention.

Further in a third aspect, the invention relates to a recombinant host cell comprising DNA isolated by a method as described in the first aspect of the invention or the vector according to the second aspect of the invention.

Also a fourth aspect relates to a transgenic animal containing and expressing DNA isolated by a method as defined in the first aspect of the invention.

A fifth aspect is a transgenic plant containing and expressing DNA isolated by a method as defined in the first aspect of the invention.

A sixth aspect relates to a method of producing a material of interest, which method comprises cultivating a cell according to the third aspect in suitable culture medium under conditions permitting expression of the DNA of interest and recovering the resulting material from the culture medium.

Further a seventh aspect relates to a method of producing a material of interest, which method comprises recovering the material from any part or secrete of/from the transgenic animal according to the fourth aspect.

In a final aspect the invention relates to a method of producing a material of interest, which method comprises growing a cell of a transgenic plant according to the fifth aspect, and recovering the material from the resulting plant.

Definitions

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (herein "Sambrook et al., 1989") *DNA Cloning: A Practical Approach*, Volumes I and II /D.N. Glover ed. 1985); *Oligonucleotide Synthesis* (M.J. Gait ed. 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins eds (1985)); *Transcription And Translation* (B.D. Hames & S.J. Higgins, eds. (1984)); *Animal Cell Culture* (R.I. Freshney, ed. (1986)); *Immobilized Cells And Enzymes* (IRL Press, (1986)); B. Perbal, *A Practical Guide To Molecular Cloning* (1984).

Means: In the first aspect of the invention, means for correlating host cell secretion of a material of interest in a sample with the fluorescence of the sample are to be established. These means are to be understood as a physical link between the sample and a fluorescent label, such as a chemical bond, an

electrostatic bond, or a lipohilic anchoring of a lipohilic molecule carrying a fluorescent label to a cell membrane of the sample. Detection of the material of interest being produced in a sample may depend on the breakage of such a physical link, whereupon the fluorescent label may diffuse out and away from the sample.

"-": When applied to e.g. a substrate construct of the structure: agarose-(DTAF)-substrate-rhodamine, the "-" indicates a covalent bond between the agarose and DTAF, DTAF and the substrate, and the substrate and rhodamine.

Isolated: When applied to a protein, the term "isolated" indicates that the protein is found in a condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the proteins in a highly purified form, i.e., greater than 95% pure, more preferably greater than 99% pure. When applied to a polynucleotide molecule, the term "isolated" indicates that the molecule is removed from its natural genetic milieu, and is thus free of other extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are ordinarily associated, and may include naturally occurring 5′ and 3′ untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, Nature 316: 774-78, 1985).

A "polynucleotide" is a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5′ to the 3′ end. Polynucleotides include RNA and DNA, and may be

isolated from natural sources, synthesized *in vitro*, or prepared from a combination of natural and synthetic molecules.

A "nucleic acid molecule" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary or quaternary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA). A "recombinant DNA molecule" is a DNA molecule that has undergone a molecular biological manipulation.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide in a cell *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. If the coding sequence is intended for expression in a eukaryotic cell, a polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

Host Cells

The present invention relates to host cells and recombinant host cells, comprising a DNA sequence of interest, or nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the material of interest. The term "host cell" encompasses any progeny of a parent cell which is not identical to the parent cell due to mutations that occur during replication.

The cell is preferably transformed with a vector comprising a nucleic acid sequence of the invention followed by integration of the vector into the host chromosome.

Transformation means introducing a vector comprising a nucleic acid sequence of the present invention into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the host chromosome may occur by homologous or non-homologous recombination as described above.

The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source. The host cell may be a unicellular microorganism, *e.g.*, a prokaryote, or a non-unicellular microorganism, e.g., a eukaryote. Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus clausii*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp.

The transformation of a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*,

Chang and Cohen, 1979, *Molecular General Genetics* 168:111-115), by using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81:823-829, or Dubnar and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56:209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6:742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169:5771-5278).

The host cell may be a eukaryote, such as a mammalian cell, an insect cell, a plant cell or a fungal cell.

Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of other immortalized cell lines available, *e.g.*, from the American Type Culture Collection.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650 and 1651), BHK (ATCC CRL 1632, 10314 and 1573, ATCC CCL 10), CHL (ATCC CCL39) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159 (1982), 601 - 621; Southern and Berg, J. Mol. Appl. Genet. 1 (1982), 327 - 341; Loyter et al., Proc. Natl. Acad. Sci. USA 79 (1982), 422 - 426; Wigler et al., Cell 14 (1978), 725; Corsaro and Pearson, Somatic Cell Genetics 7 (1981), 603, Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Inc., N.Y., 1987, Hawley-Nelson et al., Focus 15 (1993), 73; Ciccarone et al., Focus 15 (1993), 80; Graham and van der Eb, Virology 52 (1973), 456; and Neumann et al., EMBO J. 1 (1982), 841 - 845.

The host cell may be a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*, *Eupenicillium* (=*Penicillium*), *Emericella*

(=*Aspergillus*), *Eurotium* (=*Aspergillus*), and the true yeasts listed above. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, *e.g.*, *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, *e.g.*, Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Aspergillus*, *Penicillium*, *Candida*, and *Alternaria*. Representative groups of Zygomycota include, *e.g.*, *Rhizopus* and *Mucor*.

A fungal host cell may also be a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (*e.g.*, genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (*e.g.*, genera *Pichia*, *Kluyveromyces* and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Leucosporidim*, *Rhodosporidium*, *Sporidiobolus*, *Filobasidium*, and *Filobasidiella*. Yeast belonging to the Fungi Imperfecti is divided into two families, Sporobolomycetaceae (*e.g.*, genera *Sorobolomyces* and *Bullera*) and Cryptococcaceae (*e.g.*, genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics is well known in the art (see, *e.g.*, *Biochemistry and Genetics of Yeast*, Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors, 2nd edition, 1987; *The Yeasts*, Rose, A.H., and Harrison, J.S., editors, 2nd edition, 1987; and *The Molecular Biology of the Yeast Saccharomyces*, Strathern *et al.*, editors, 1981).

The yeast host cell may be selected from a cell of a species of *Candida*, *Kluyveromyces*, *Saccharomyces*, *Schizosaccharo-*

myces, *Candida*, *Pichia*, *Hansehula*, or *Yarrowia*. Useful yeast host cells are *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. Other useful yeast host cells are a *Kluyveromyces lactis Kluyveromyces fragilis Hansehula polymorpha*, *Pichia pastoris Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Ustilgo maylis*, *Candida maltose*, *Pichia guillermondii and Pichia methanolio cell* (cf. Gleeson et al., J. Gen. Microbiol. 132, 1986, pp. 3459-3465; US 4,882,279 and US 4,879,231).

The fungal host cell may be a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a vegetative mycelium composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. The filamentous fungal host cell can be a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, and *Trichoderma* or a teleomorph or synonym thereof.

Particularly useful filamentous fungal host cells are *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger*, *Aspergillus nidulans* or *Aspergillus oryzae*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023.

Transformation

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceed-*

ings of the National Academy of Sciences USA 81:1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78:147-156. Examples of other fungal cells are cells of filamentous fungi, e.g. *Aspergillus* spp., *Neurospora* spp., *Fusarium* spp. or *Trichoderma* spp., in particular strains of *A. oryzae*, *A. nidulans* or *A. niger*. The use of *Aspergillus* spp. for the expression of proteins is described in, e.g., EP 272 277, EP 230 023.

Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153:163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:1920. Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb (1978, *Virology* 52:546).

Transformation of insect cells and production of heterologous polypeptides therein may be performed as described in US 4,745,051; US 4, 775, 624; US 4,879,236; US 5,155,037; US 5,162,222; EP 397,485) all of which are incorporated herein by reference. The insect cell line used as the host may suitably be a *Lepidoptera* cell line, such as *Spodoptera frugiperda* cells or *Trichoplusia ni* cells (cf. US 5,077,214). Culture conditions may suitably be as described in, for instance, WO 89/01029 or WO 89/01028, or any of the aforementioned references.

A recombinant vector

One aspect of the present invention relates to a recombinant vector comprising DNA isolated by a method as defined in any of the preceding aspects.

The recombinant vector into which the DNA of the invention is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously

replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

The vector is preferably an expression vector in which the DNA sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the DNA. In general, the expression vector is derived from plasmid or viral DNA, or may contain elements of both. The term, "operably linked" indicates that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in a promoter and proceeds through the DNA sequence coding for the polypeptide.

The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the DNA encoding the polypeptide of the invention in mammalian cells are the SV40 promoter (Subramani et al., Mol. Cell Biol. 1 (1981), 854 -864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222 (1983), 809 - 814) or the adenovirus 2 major late promoter.

An example of a suitable promoter for use in insect cells is the polyhedrin promoter (US 4,745,051; Vasuvedan et al., FEBS Lett. 311, (1992) 7 - 11), the P10 promoter (J.M. Vlak et al., J. Gen. Virology 69, 1988, pp. 765-776), the *Autographa californica* polyhedrosis virus basic protein promoter (EP 397 485), the baculovirus immediate early gene 1 promoter (US 5,155,037; US 5,162,222), or the baculovirus 39K delayed-early gene promoter (US 5,155,037; US 5,162,222).

Examples of suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255 (1980), 12073 - 12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1 (1982), 419 - 434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4,599,311) or ADH2-4c (Russell et al., Nature 304 (1983), 652 - 654) promoters.

Examples of suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4 (1985), 2093 - 2099) or the tpiA promoter. Examples of other useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral a-amylase, *A. niger* acid stable a-amylase, *A. niger* or *A. awamori* glucoamylase (gluA), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and gluA promoters.

Examples of suitable promoters for use in bacterial host cells include the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene, the *Bacillus licheniformis* alpha-amylase gene, the *Bacillus amyloliquefaciens* BAN amylase gene, the *Bacillus subtilis* alkaline protease gene, or the *Bacillus pumilus* xylosidase gene, or by the phage Lambda P_R or P_L promoters or the E. coli lac, trp or tac promoters.

The DNA of the invention may also, if necessary, be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) terminators. The vector may further comprise elements such as polyadenylation signals (e.g. from SV40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant vector of the invention may further comprise a DNA sequence enabling the vector to replicate in the

host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication.

When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2m replication genes REP 1-3 and origin of replication.

When the host cell is a bacterial cell, sequences enabling the vector to replicate are *legio* in the art

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g. ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For filamentous fungi, selectable markers include amdS, pyrG, argB, niaD, and sC.

To direct a polypeptide encoded by the DNA of the present invention into the secretory pathway of the host cells, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) may be provided in the recombinant vector. The secretory signal sequence is joined to the DNA sequence encoding the polypeptide in the correct reading frame. Secretory signal sequences are commonly positioned 5' to the DNA sequence encoding the polypeptide. The secretory signal sequence may be that normally associated with the polypeptide or may be from a gene encoding another secreted protein.

For secretion from yeast cells, the secretory signal sequence may encode any signal peptide ensuring efficient direction of the expressed polypeptide into the secretory pathway of the cell. The signal peptide may be naturally occurring signal peptide, or a functional part thereof, or it may be a synthetic peptide. Suitable signal peptides have been found to be the a-factor signal peptide (cf. US 4,870,008), the signal peptide of mouse salivary amylase (cf. O. Hagenbuchle et

al., Nature 289, 1981, pp. 643-646), a modified carboxypeptidase signal peptide (cf. L.A. Valls et al., Cell 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), or the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., Yeast 6, 1990, pp. 127-137).

For efficient secretion in yeast, a sequence encoding a leader peptide may also be inserted downstream of the signal sequence and uptream of the DNA sequence encoding the polypeptide. The function of the leader peptide is to allow the expressed polypeptide to be directed from the endoplasmic reticulum to the Golgi apparatus and further to a secretory vesicle for secretion into the culture medium (i.e. exportation of the polypeptide across the cell wall or at least through the cellular membrane into the periplasmic space of the yeast cell). The leader peptide may be the yeast a-factor leader (the use of which is described in e.g. US 4,546,082, EP 123 294, EP 123 544 and EP 163 529). Alternatively, the leader peptide may be a synthetic leader peptide, which is to say a leader peptide not found in nature. Synthetic leader peptides may, for instance, be constructed as described in WO 89/02463 or WO 92/11378.

For use in filamentous fungi, the signal peptide may conveniently be derived from a gene encoding an *Aspergillus* sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease, or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral a-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase.

For use in insect cells, the signal peptide may conveniently be derived from an insect gene (cf. WO 90/05783), such as the lepidopteran *Manduca sexta* adipokinetic hormone precursor signal peptide (cf. US 5,023,328).

The procedures used to ligate the DNA of the invention, the promoter and optionally the terminator and/or secretory signal sequence, respectively, and to insert them into suitable

vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

Transgenic animals

It is also within the scope of the present invention to employ transgenic animal technology to produce the present material. A transgenic animal is one in whose genome a heterologous DNA sequence has been introduced. In particular, a polypeptide of the invention may be expressed in the mammary glands of a non-human female mammal, in particular one which is known to produce large quantities of milk. Examples of preferred mammals are livestock animals such as goats, sheep and cattle, although smaller mammals such as mice, rabbits or rats may also be employed.

The DNA sequence of interest may be introduced into the animal by any one of the methods previously described for the purpose. For instance, to obtain expression in a mammary gland, a transcription promoter from a milk protein gene is used. Milk protein genes include the genes encoding casein (cf. US 5,304,489), beta-lactoglobulin, alpha-lactalbumin and whey acidic protein. The currently preferred promoter is the beta-lactoglobulin promoter (cf. Whitelaw et al., Biochem J. 286, 1992, pp. 31-39).

It is generally recognized in the art that DNA sequences lacking introns are poorly expressed in transgenic animals in comparison with those containing introns (cf. Brinster et al., Proc. Natl. Acad. Sci. USA 85, 1988, pp. 836-840; Palmiter et al., Proc. Natl. Acad. Sci. USA 88, 1991, pp. 478-482; Whitelaw et al., Transgenic Res. 1, 1991, pp. 3-13; WO 89/01343; WO 91/02318). For expression in transgenic animals, it is therefore preferred, whenever possible, to use genomic sequences containing all or some of the native introns of the DNA of interest. It may also be preferred to include at least some introns from, e.g. the beta-lactoglobulin gene. One such region is a DNA segment which provides for intron splicing and

RNA polyadenylation from the 3' non-coding region of the ovine beta-lactogloblin gene. When substituted for the native 3' non-coding sequences of a gene, this segment may will enhance and stabilise expression levels of the polypeptide of interest. It may also be possible to replace the region surrounding an initiation codon with corresponding sequences of a milk protein gene. Such replacement provides a putative tissue-specific initiation environment to enhance expression.

For expression of the DNA of interest in transgenic animals it is operably linked to additional DNA sequences required for its expression to produce expression units. Such additional sequences include a promoter as indicated above, as well as sequences providing for termination of transcription and polyadenylation of mRNA. The expression unit further includes a DNA sequence encoding a secretory signal sequence operably linked to the sequence encoding the polypeptide. The secretory signal sequence may be one native to the polypeptide or may be that of another protein such as a milk protein (cf. von Heijne et al., Nucl. Acids Res. 14, 1986, pp. 4683-4690; and US 4,873,316).

Construction of the expression unit for use in transgenic animals may conveniently be done by inserting the DNA sequence of interest into a vector containing the additional DNA sequences, although the expression unit may be constructed by essentially any sequence of ligations. It is particularly convenient to provide a vector containing a DNA sequence encoding a milk protein and to replace the coding region for the milk protein with the DNA sequence of interest, thereby creating a fusion which includes expression control sequences of the milk protein gene.

The expression unit is then introduced into fertilized ova or early-stage embryos of the selected host species. Introduction of heterologous DNA may be carried out in a number of ways, including microinjection (cf. US 4,873,191), retroviral infection (cf. Jaenisch, Science 240, 1988, pp. 1468-1474) or site-directed integration using embryonic stem

cells (reviewed by Bradley et al., Bio/Technology 10, 1992, pp. 534-539). The ova are then implanted into the oviducts or uteri of pseudopregnant females and allowed to develop to term. Offspring carrying the introduced DNA in their germ line can pass the DNA on to their progeny, allowing the development of transgenic herds.

General procedures for producing transgenic animals are known in the art, cf. for instance, Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986; Simons et al., Bio/Technology 6, 1988, pp. 179-183; Wall et al., Biol. Reprod. 32, 1985, pp. 645-651; Buhler et al., Bio/Technology 8, 1990, pp. 140-143; Ebert et al., Bio/Technology 6: 179-183, 1988; Krimpenfort et al., Bio/Technology 9: 844-847, 1991, Wall et al., J. Cell. Biochem. 49: 113-120, 1992; US 4,873,191, US 4,873,316; WO 88/00239, WO 90/05188; WO 92/11757 and GB 87/00458. Techniques for introducing heterologous DNA sequences into mammals and their germ cells were originally developed in the mouse. See, e.g. Gordon et al., Proc. Natl. Acad. Sci. USA 77: 7380-7384, 1980, Gordon and Ruddle, Science 214: 1244-1246, 1981; Palmiter and Brinster, Cell 41: 343-345, 1985; Brinster et al., Proc. Natl. Acad. Sci. USA 82: 4438-4442, 1985; and Hogan et al. (ibid.). These techniques were subsequently adapted for use with larger animals, including livestock species (see e.g., WO 88/00239, WO 90/01588 and WO 92/11757; and Simons et al., Bio/Technology 6: 179-183, 1988). To summarize, in the most efficient route used to date in the generation of transgenic mice or livestock, several hundred linear molecules of the DNA of interest are injected into one of the pro-nuclei of a fertilized egg according to techniques which have become standard in the art. Injection of DNA into the cytoplasm of a zygote can also be employed.

Transgenic plants

Production in transgenic plants may also be employed. It has previously been described to introduce DNA sequences into

plants, which sequences code for protein products imparting to the transformed plants certain desirable properties such as increased resistance against pests, pathogens, herbicides or stress conditions (cf. for instance EP 131 620, EP 205 518, EP 270 355, WO 89/04371 or WO 90/02804), or an improved nutrient value of the plant proteins (cf. for instance EP 90 033, EP 205 518 or WO 89/04371). Furthermore, WO 89/12386 discloses the transformation of plant cells with a gene coding for levansucrase or dextransucrase, regeneration of the plant (especially a tomato plant) from the cell resulting in fruit products with altered viscosity characteristics.

In the plant cell, the DNA of interest is under the control of a regulatory sequence which directs the expression of the DNA sequence in plant cells and intact plants. The regulatory sequence may be either endogenous or heterologous to the host plant cell.

The regulatory sequence may comprise a promoter capable of directing the transcription of the DNA sequence of interest in plants. Examples of promoters which may be used according to the invention are the 35s RNA promoter from cauliflower mosaic virus (CaMV), the class I patatin gene B 33 promoter, the ST-LS1 gene promoter, promoters conferring seed-specific expression, e.g. the phaseolin promoter, or promoters which are activated on wounding, such as the promoter of the proteinase inhibitor II gene or the wun1 or wun2 genes.

The promoter may be operably connected to an enhancer sequence, the purpose of which is to ensure increased transcription of the DNA of interest. Examples of useful enhancer sequences are enhancers from the 5'-upstream region of the 35s RNA of CaMV, the 5'-upstream region of the ST-LS1 gene, the 5'-upstream region of the Cab gene from wheat, the 5'-upstream region of the 1'- and 2'-genes of the T_R-DNA of the Ti plasmid pTi ACH5, the 5'-upstream region of the octopine synthase gene, the 5'-upstream region of the leghemoglobin gene, etc.

The regulatory sequence may also comprise a terminator capable of terminating the transcription of the DNA of interest in plants. Examples of suitable terminators are the terminator of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5 of Agrobacterium tumefaciens, of the gene 7 of the T-DNA of the Ti plasmid pTiACH5, of the nopaline synthase gene, of the 35s RNA-coding gene from CaMV or from various plant genes, e.g. the ST-LS1 gene, the Cab gene from wheat, class I and class II patatin genes, etc.

The DNA of interest may also be operably connected to a DNA sequence encoding a leader peptide capable of directing the transport of an expressed polypeptide to a specific cellular compartment (e.g. vacuoles) or to extracellular space. Examples of suitable leader peptides are the leader peptide of proteinase inhibitor II from potato, the leader peptide and an additional about 100 amino acid fragments of patatin, or the transit peptide of various nucleus-encoded proteins directed into chloroplasts (e.g. from the St-LS1 gene, SS-Rubisco genes, etc.) or into mitochondria (e.g. from the ADP/ATP translocator).

Furthermore, the DNA of interest may be modified in the 5' non-translated region resulting in enhanced translation of the sequence. Such modifications may, for instance, result in removal of hairpin loops in RNA of the 5' non-translated region. Translation enhancement may be provided by suitably modifying the omega sequence of tobacco mosaic virus or the leaders of other plant viruses (e.g. BMV, MSV) or of plant genes expressed at high levels (e.g. SS-Rubisco, class I patatin or proteinase inhibitor II genes from potato).

The DNA of interest may furthermore be connected to a second DNA sequence encoding another polypeptide or a fragment thereof in such a way that expression of said DNA sequences results in the production of a fusion protein. When the host cell is a potato plant cell, the second DNA sequence may, for instance, encode patatin or a fragment thereof (such as a fragment of about 100 amino acids).

The plant in which the DNA of interest is introduced may suitably be a dicotyledonous plant, examples of which are is a tobacco, potato, tomato, or leguminous (e.g. bean, pea, soy, alfalfa) plant. It is, however, contemplated that monocotyledonous plants, e.g. cereals, may equally well be transformed with the DNA.

Procedures for the genetic manipulation of monocotyledonous and dicotyledonous plants are well known. In order to construct foreign genes for their subsequent introduction into higher plants, numerous cloning vectors are available which generally contain a replication system for E. coli and a selectable/screenable marker system permitting the recognition of transformed cells. These vectors include e.g. pBR322, the pUC series, pACYC, M13 mp series etc. The foreign sequence may be cloned into appropriate restriction sites. The recombinant plasmid obtained in this way may subsequently be used for the transformation of E. coli. Transformed E. coli cells may be grown in an appropriate medium, harvested and lysed. The chimeric plasmid may then be reisolated and analyzed. Analysis of the recombinant plasmid may be performed by e.g. determination of the nucleotide sequence, restriction analysis, electrophoresis and other molecular-biochemical methods. After each manipulation the sequence may be cleaved and ligated to another DNA sequence. Each DNA sequence can be cloned on a separate plasmid DNA. Depending on the way used for transferring the foreign DNA into plant cells other DNA sequences might be of importance. In case the Ti-plasmid or the Ri plasmid of *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, at least the right border of the T-DNA may be used, and often both the right and the left borders of the T-DNA of the Ri or Ti plasmid will be present flanking the DNA sequence to be transferred into plant cells.

The use of the T-DNA for transferring foreign DNA into plant cells has been described extensively in the prior literature (cf. Gasser and Fraley, 1989, Science 244, 1293 - 1299 and references cited therein). After integration of the

foreign DNA into the plant genome, this sequence is fairly stable at the original locus and is usually not lost in subsequent mitotic or meiotic divisions. As a general rule, a selectable marker gene will be co-transferred in addition to the gene to be transferred, which marker renders the plant cell resistant to certain antibiotics, e.g. kanamycin, hygromycin, G418 etc. This marker permits the recognition of the transformed cells containing the DNA sequence to be transferred compared to non-transformed cells.

Numerous techniques are available for the introduction of DNA into a plant cell. Examples are the *Agrobacterium* mediated transfer, the fusion of protoplasts with liposomes containing the respective DNA, microinjection of foreign DNA, electroporation etc. In case *Agrobacterium* mediated gene transfer is employed, the DNA to be transferred has to be present in special plasmids which are either of the intermediate type or the binary type. Due to the presence of sequences homologous to T-DNA sequences, intermediate vectors may integrate into the Ri- or Ti-plasmid by homologous recombination. The Ri- or Ti-plasmid additionally contains the vir-region which is necessary for the transfer of the foreign gene into plant cells. Intermediate vectors cannot replicate in *Agrobacterium* species and are transferred into *Agrobacterium* by either direct transformation or mobilization by means of helper plasmids (conjugation). (Cf. Gasser and Fraley, op. cit. and references cited therein).

Binary vectors may replicate in both *Agrobacterium* species and E. coli. They may contain a selectable marker and a poly-linker region which to the left and right contains the border sequences of the T-DNA of *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens*. Such vectors may be transformed directly into *Agrobacterium* species. The *Agrobacterium* cell serving as the host cell has to contain a vir-region on another plasmid. Additional T-DNA sequences may also be contained in the *Agrobacterium* cell.

The *Agrobacterium* cell containing the DNA sequences to be transferred into plant cells either on a binary vector or in the form of a cointegrate between the intermediate vector and the T-DNA region may then be used for transforming plant cells. Usually either multicellular explants (e.g. leaf discs, stem segments, roots), single cells (protoplasts) or cell suspensions are co-cultivated with Agrobacterium cells containing the DNA sequence to be transferred into plant cells. The plant cells treated with the *Agrobacterium* cells are then selected for the co-transferred resistance marker (e.g. kanamycin) and subsequently regenerated to intact plants. These regenerated plants will then be tested for the presence of the DNA sequences to be transferred.

If the DNA is transferred by e.g. electroporation or microinjection, no special requirements are needed to effect transformation. Simple plasmids e.g. of the pUC series may be used to transform plant cells. Regenerated transgenic plants may be grown normally in a greenhouse or under other conditions. They should display a new phenotype (e.g. production of new proteins) due to the transfer of the foreign gene(s). The transgenic plants may be crossed with other plants which may either be wild-type or transgenic plants transformed with the same or another DNA sequence. Seeds obtained from transgenic plants should be tested to assure that the new genetic trait is inherited in a stable Mendelian fashion.

See also Hiatt, Nature 344: 469-479, 1990; Edelbaum et al., J. Interferon Res. 12: 449-453, 1992; Sijmons et al., Bio/Technology 8: 217-221, 1990: and EP 255 378.

Brief Description of the Figure

Figure 1 A-B shows the results of a Fluorescence Activated Cell Sorter (FACS) analysis using a FACSCalibur (Becton Dickinson, USA). Figure 1A shows the distribution of fluorescence of Fluorescein labeled Gel Micro Drops (F-GMD's), and Figure 1B shows the quenching achieved by addition of anti-fluorescein Antibodies to the F-GMD's. Gates R1 and R2 repre-

sent fluorescent and quenched F-GMD's respectively. Figure 1C shows that addition of 5µl protease (Alcalase®, Novo Nordisk, Denmark) shifts the F-GMD's into R1, thus showing that fluorescence is recovered, or de-quenching has occurred. Figure 1D shows a FACS-analysis of a mixture of 99% quenched F-GMD's and 1% protease de-quenched F-GMD's. Approximately 0.7% of the gated F-GMD's were located in R1, thus demonstrating the sensitivity of the FACS-analysis for even as low a number of F-GMD's as 1%.

Detailed description of the invention

In a first embodiment the invention relates to a method for screening a DNA library for DNA of interest comprising the steps of a) creating host cells comprising the DNA library, b) generating samples each comprising a host cell of step a), c) establishing a means for correlating host cell secretion of a material of interest in a sample with the fluorescence of the sample, d) determining which intensity interval of fluorescence indicates secretion in the sample when the correlating means of step c) is used, e) cultivating the samples under suitable conditions, and f) selecting the samples exhibiting fluorescence within the intensity interval of step d) using a fluorescence analyzer; wherein the host cell comprises DNA of interest.

In the art, ways of generating and producing DNA libraries from natural sources are well known, but besides natural DNA sequences, a number of ways are also known in which to generate very large populations of diverse artificial DNA sequences starting from one or more natural sequences, e.g. shuffling or directed evolution (WO 98/42832; US 5,965,408; WO 98/01581; WO 97/07205; WO 95/22625; US 5,093,257).

Preferably the DNA library is generated from a natural DNA sequence by DNA shuffling or directed evolution.

When attempting to express a DNA library, it is a matter of choice, in which host cell to do so.

A preferred embodiment of the invention relates to a method of the first aspect, wherein the host cells of the first aspect step a) are of bacterial or fungal origin.

The host cells of the first aspect step a) of the invention may be any bacterial or fungal cells, non-limiting examples of which are *Bacillus lentus, Bacillus subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus agaradherens, Bacillus alkalophilus, Bacillus clausii, Bacillus circulans, Bacillus firmus, Bacillus thuringiensis, Aspergillus oryzae, Aspergillus niger,* and *Aspergillus aculateus.*

Preferably the host cells of the first aspect step a) are *Escherichia coli, Bacillus subtilis* or *Aspergillus oryzae.*

The host cells could be labeled directly with a fluorescent substrate that is converted by the material of interest, which would make the labeled cells the actual samples of the first aspect of the invention step b). Fluorescein labeled substrate could be coupled directly to the cell surface using lipid or electrostatic anchoring or simply covalent bonding. When the cell secretes a material of interest, the substrate is converted and the fluorescence may be de-quenched, lost, or it may change polarization, depending on the assay principle applied.

Below are given some principle methods of to attach the labeled substrate to the cell: a) Lipid anchoring of a label tagged with a lipophilic tail which anchors in the cell membrane; b) Fluorescein labeled Pectin tagged with a C18 lipophillic tail which anchors in the cell membrane; c) Covalent bonding by fluorescein labeled substrate, which is synthesized with an activated reducing end, and upon contact with the cell the labeled substrate is covalently bound to amino-groups on the cell surface; d) Electrostatic binding (WO 98/49286); e) A labeled substrate comprising a maltose which binds to the phage lambda receptor on the cell surface. These approaches can be applied to any of the assay principles mentioned in examples 1-5.

The art teaches ways of cultivating monoclonal microcolonies of bacterial or fungal cells within micro-environments, which can then be analyzed using a flow cytometer. One example of such monoclonal microcolony cultivation within a microenvironment is described in (Sahar, E., R. Nir, and R. Lamed, 1994. Flow cytometric analysis of entire microbial colonies. *Cytometry* 15:213-221).

A further embodiment of the invention relates to a method of the first aspect, wherein the samples of the first aspect step b) are microenvironments.

A number of different closed microenvironments have been described in the art (WO 98/41869), (Nir, R., et al., 1990, Single-cell entrapment and microcolony development within uniform microspheres amenable to flow cytometry. *Appl. Environ. Microbiol.* 56:2870-2875), (WO 98/58085). Closed micro-environments, such as liposomes, beads, cells, ghost red blood cells, or ghost macrophages have the advantage that the very little diffusion occurs from/to them, which allows the means for correlation of the first aspect of the invention step c), to be chosen more broadly. The material of interest does not diffuse away from the micro-environment and any fluorescent signal will stay within, so the correlation between secreted material and fluorescence is easier maintained in a closed micro-environment.

Preferably the micro-environments are liposomes, beads, cells, ghost red blood cells, or ghost macrophages.

As mentioned previously a wide array of micro-environments have been described in the art, the majority of these are of the open kind, such as gel micro drops comprising agarose, polysaccharide, carbohydrate, alginate, carrageenan, chitosan, cellulose, pectin, dextran or polyacrylamide, allowing diffusion to/from each micro-environment. However these open micro-environments are functional in the present invention, when applying the means for correlation of the first aspect of the invention step c).

Accordingly preferred micro-environments are gel micro drops (GMD's) comprising agarose, polysaccharide, carbohydrate, alginate, carrageenan, chitosan, cellulose, pectin, dextran or polyacrylamide.

There are some difficulties associated with growing filamentous fungi in GMD's (see example 6), however using high molecular weight dextran in the GMD's as essentially the sole carbon source can alleviates some of these difficulties.

Preferably the GMD's comprise as the essentially sole carbon source high molecular weight (HMW) dextran.

As mentioned previously, when screening micro-environments for cells that secrete a certain material of interest, it is necessary to be able to correlate the presence of said material fairly accurately with whatever signal is measured in the screening assay. This can be a problem if the material of interest is highly diffusible in the microenvironments, the material may simply diffuse out into the surrounding liquid, or there may be inter-microenvironment diffusion. Closed or compartmentalized micro-environments would solve this diffusion problem.

Compartmentalization could be done by suspending the micro-environments in a non-aqueous phase (e.g. mineral oil or emulsion oil) and allow them to incubate until sufficient growth and secretion had occurred to allow detection. The micro-environments could then be subjected to flow cytometry (e.g. using a nano-FACS) with the hydrophobic liquid as carrier fluid (sheet fluid) ensuring that they remained compartmentalized.

Several ways of solving such diffusion problems have been described in the art (US 4,401,755), particularly focusing on ways of coating the microenvironments with a secondary layer through which diffusion of the material of interest is insignificant, e.g. a thin polymer film with a certain molecular cut off, or a double lipid layer. Ideally oxygen, and growth substrate should diffuse in while substrate and fluorescence degradation products should not diffuse out.

Preferably the micro-environments are encapsulated by a layer, said layer being essentially non-penetrable to the material of interest, preferably the layer comprises a lipid material.

As discussed above, some of the industrially important products that are manufactured by fermenting microbes are proteins or polypeptides.

Consequently a preferred embodiment of the invention relates to a method of the first aspect, wherein the material of interest of the first aspect step c) is a polypeptide.

Of the polypeptides especially the industrial enzymes are produced in a large scale.

Preferably the polypeptide is an enzyme selected from the group consisting of lipases, esterases, proteases, glucosidases, glucosyl transferases, phosphatases, phytases, kinases, mono- and dioxygenases, haloperoxidases, peroxidases, transaminases, rhamnogalacturonases, lactases, and laccases.

However polypeptides need not necessarily be enzymes, other products of interest are polypeptides that interfere with microbial metabolism or peptides that have pharmaceutical properties such as peptide hormones or neuropeptides.

Preferably the polypeptide is an antimicrobial agent, a growth-promoting agent, or a pharmaceutical peptide.

Isolating DNA of interest and expressing this DNA in a host cell can lead to the production of a polypeptide with an intracellular activity, which in turn may give rise to the production and/or secretion of various molecules or compounds, some of which may have pharmaceutical properties.

Accordingly a preferred embodiment is a method according to the first aspect, wherein the material of interest of the first aspect step c) is a pharmaceutical compound.

As mentioned above, some of the difficulties in screening host cells for secretion of a material of interest are due to diffusion of the material away from the secreting host cell. In order to identify a sample comprising a secreting host cell, a physical means must be established for correlating host cell secretion with a detectable property. As a Fluorescence Acti-

vated Cell Sorter is well known in the art, basing the beforementioned means for correlation based on a fluorescent label would be advantageous (see examples 1-5, and 7).

Consequently, a preferred embodiment is a method according to the first aspect, wherein the means of the first aspect step c) is based on labelling the sample with a fluorescent group, preferably fluorescein, more preferably dichlorotriazino-5-amino-fluorescein (DTAF).

Having a fluorescently labeled sample further allows several screening strategies based on measuring disappearance of fluorescence caused by quenching or Fluorescence Resonance Energy Transfer (FRET), or measuring the re-appearance of fluorescence caused by de-quenching; some non-limiting examples are shown below (see examples 1-5, and 7).

Preferably the means of the first aspect step c) is further based on Fluorescence Resonance Energy Transfer (FRET), quenching of the fluorescent group, or de-quenching of the fluorescent group.

Several groups are known in the art to cause fluorescence quenching or FRET, some of these are shown as non-limiting examples herein (see examples 1-5, and 7).

Accordingly a preferred embodiment is a method according to the first aspect, wherein the FRET, quenching or de-quenching is achieved by using a polypeptide, preferably rhodamine, more preferably hemoglobin, even more preferably casein, and most preferably antibodies directed towards the fluorescent group.

Bi-functional dyes are capable of forming two covalent bonds, a specific bi-functional fluorescent dye is dichlorotriazino-5-amino-fluorescein (DTAF). Such dyes can be cross-linked to a polymer anchoring material comprised in a sample of the first aspect, such as agarose (see example 1). Further, they can also cross-link the polymer with a quenching group, such as a protein like hemoglobin (see example 3) or casein.

A further preferred embodiment is a method according to the first aspect, wherein the material of interest is a prote-

ase, and wherein the means of the first aspect step c) is based on labelling the sample with a bi-functional fluorescent dye, and where the fluorescence of said dye is quenched by anti-fluorescent antibodies, or by a protein covalently linked to the dye, preferably the bi-functional dye is dichlorotriazino-5-amino-fluorescein (DTAF).

One exemplified protease screening strategy involves labelling the sample of the first aspect step b) with biotin, conjugating all free biotin with avidin, a protease-substrate, and then titrating newly freed biotin in the sample with fluorescently labeled avidin to establish the amount of unlabeled avidin degraded (see example 2).

Accordingly a preferred embodiment is a method according to the first aspect, wherein the material of interest is a protease, and wherein the means of claim 1 step c) is based on labelling the sample with biotin and conjugating the biotin with fluorescently labeled avidin or streptavidin.

The invention is also exemplified in a non-limiting way in a screening strategy for DNA encoding amylase (see example 4).

Accordingly a preferred embodiment is a method according to the first aspect, wherein the material of interest is an amylase, and wherein the means of the first aspect step c) is based on quenching/de-quenching or FRET in a substrate construct having the following structure: agarose-(bi-functional fluorescent dye)-substrate-rhodamine, where the substrate is at least in part degraded by the amylase thereby effectively releasing the rhodamine.

One non-limiting exemplified amylase screening strategy (see example 5) involves labelling the sample of the first aspect step b) with a fluorescent degradable substrate, which in its undegraded form is non-diffusable within the sample. The substrate, when degraded by an active amylase, diffuses out of the sample thereby effectively lowering the fluorescence of the sample. In example 5 we describe the use of crosslinked starch in microspheres, however the concept of a non-diffusable but

degradable substrate can be generally applied, e.g. by using cross-linked proteins when screening for proteases.

A preferred embodiment is a method according to the first aspect, wherein the means of the first aspect step c) is based on a fluorescently labeled degradable substrate which when present in the samples of the first aspect step b) is non-diffusable in its un-degraded form and diffusable when degraded, preferably the substrate is fluorescently labeled substrate microspheres, preferably starch microspheres.

In a non-limiting fashion, it is shown (see example 7) that the present invention also relates to the screening of antimicrobial peptides.

Another preferred embodiment is a method according to the first aspect of the invention, wherein the material of interest is an anti-microbial peptide, and where the samples of the first aspect step b) further comprise a tester cell that displays a detectable response to the anti-microbial peptide.

The present invention includes within its embodiments any apparatus capable of detecting fluorescent wavelengths associated with biological material as well as fluorescence intensity and/or fluorescence polarization or anisitropy, such apparatii are defined herein as fluorescent analyzers, one example of which is a Fluorescence Activated Cell Sorter (FACS).

Accordingly a preferred embodiment is the method according to the first aspect, wherein the fluorescence analyzer step f) is a Fluorescence Activated Cell Sorter (FACS).

A recombinant vector according to the second aspect comprising DNA isolated by a method as defined in the first aspect of the invention.

A recombinant host cell according to the third aspect comprising DNA isolated by a method as described in the first aspect of the invention or the vector according to the second aspect of the invention.

As mentioned in the description of the main aspects of the invention, several types of host cells can be utilized in the methods of the invention.

A preferred embodiment of the invention is a host cell of the third aspect of the invention, which is of microbial origin, preferably of *Escherichia, Bacillus* or *Aspergillus* origin, even more preferably chosen from the group consisting of: *Escherichia coli, Bacillus subtilis, Bacillus licheniformis, Bacillus clausii, Aspergillus niger, Aspergillus oryzae,* and *Aspergillus nidulans*.

A transgenic animal according to the fourth aspect containing and expressing DNA isolated by a method as defined in the first aspect of the invention.

A transgenic plant according to the fifth aspect containing and expressing DNA isolated by a method as defined in the first aspect of the invention.

The industrial interest in the method of the invention is of course spurred by the potential of producing the molecule of interest according to the aspects of the invention. Hence the invention relates to the ways known in the art of producing such molecules as described in more detail above.

A method of producing a material of interest according to the sixth aspect, which method comprises cultivating a cell according to the third aspect in suitable culture medium under conditions permitting expression of the DNA of interest and recovering the resulting material from the culture medium.

A method of producing a material of interest according to the seventh aspect, which method comprises recovering the material from any part or secrete of/from the transgenic animal according to the fourth aspect.

A method of producing a material of interest according to the final aspect, which method comprises growing a cell of a transgenic plant according to the fifth aspect, and recovering the material from the resulting plant.

Example 1

This example illustrates the FACS screening of a gene library for protease activity based on antibody quenching of

fluorescein labeled GMD's.

The library was constructed in *Escherichia coli* as described in PCT DK99/00495 (not published at filing date). The library was amplified in *E. coli* and plasmid was extracted and transformed into *Bacillus subtilis* Sha273 (WO95/10603).

Protease assay principle

The fluorescence of agarose beads labeled with fluorescein is quenched by an anti-fluorescein antibody. The action of a protease will degrade the anti-fluorescein antibody whereupon fluorescein will be de-quenched, thus allowing protease positive GMD's to be sorted on a FACS on the basis of increased fluorescence.

Quenching / de-quenching in microtiter plates

In 96-well plates, 10µl of anti-fluorescein IgG (Molecular probes, Eugene; Or) was added to 250 µl fluorescein solution in each well and fluorescence intensities were measured on a PolarStar (BMG Labtechnologies, Germany) with and without Ab addition (see table 1). A 53-fold reduction in fluorescence was shown with Ab addition. After addition of 1µl protease (alcalase, Novo Nordisk A/S, Denmark) a 33-fold de-quenching was measured, showing a high increase in fluorescence when the antibody was degraded by protease (see table 1).

Fluorescein	Fluorescein + Ab	Fold quenching	Fluorescein + Ab +alcalase	Fold de-quenching
53425	1002	53x	33417	33x

Table 1. Fluorescence of Fluorescein before and after quenching with an antibody (Ab), and after degradation of the quenching Ab (de-quenching).

FACS sorting of GMD's

Fluorescein labeling of agarose (F-agarose) was accomplished by dissolving agarose (2.65g Ultra-low Gelling temperature agarose cat #A2576 Sigma Aldrich) in water (70 ml) at 65°C,

pH was adjusted to 10.5 using 1N NaOH-solution. The temperature was lowered to 35°C. 25 mg DTAF (Dichlorotriazino-5-amino-fluorescein) was added and the reaction mixture was stirred in darkness overnight. The labeled polymer was precipitated in EtOH (600 ml), filtered, and washed (EtOH).

Fluorescein labeled GMD's (F-GMD's) were prepared by first mixing 10µl of a 0.5% F-agarose in 400µl 4% agarose (Ultra-low Gelling temperature agarose cat # A2576 Sigma Aldrich) at 80°C, and then generating the F-GMD's on a CelSys 100 Micro-Drop Maker (OneCell Systems) using the following protocol:

1) 15 ml emulsion oil (Dimethylpolysiloxane DMPS2C, Sigma Aldrich) in a scintillation vial and the melted agarose mix were equilibrated in a 40°C water bath.
2) Add 100 µl Pluronic acid (Pluronic F68 solution, Sigma Aldrich) to the agarose and let equilibrate at 40°C (3min).
3) Harvest cells by centrifugation and re-suspend in 100µl PBS (pH7.5)
4) Add 100 µl cells (adjust concentrations of cells to give less than one cell/GMD) or 100µl PBS to the molten agarose; equilibrate at 40°C (3min).
5) Add the agarose-cell mixture dropwise to the warmed emulsion oil avoiding air bubbles.
6) Emulsify using the following settings:
   2100 RPM for 1 min at room temperature
   2100 RPM for 2 min in ice bath
   1100 RPM for 10 min in ice bath
7) Harvest GMD's by dividing the encapsulation mixture in two 15ml conical tubes and carefully overlay the emulsion with 5 ml PBS and centrifuge for 10 min at 1500 RPM in a Megafuge 1.0R (Heraeus Instruments) centrifuge. A pellet should be visible
8) Remove the oil phase and the overlaying PBS. Wash the

pellet by adding the concentrated GMD suspension to a new 15 ml conical tube with 10 ml PBS. Spin for 10 min at 1500RPM in a Megafuge 1.0R (Heraeus Instruments) centrifuge. Wash step can be repeated if oil still is present.

9) Decant supernatant and re-suspend GMD's according to application protocol.

The protease assay was set up in the following way: Assay mix consisted of 20 µl F-GMD solution mixed with 10 µl 50mM Hepes (pH 8), optional 10µl anti-fluorescein antibody (Molecular Probes, anti-fluorescein, rabbit IgG fraction; catalog # A889;), optional protease (alcalase, Novo Nordisk A/S); the volume was brought to 50 µl using milliQ water. The assay mix was incubated over night.

Figure 1 shows the result of the FACS analysis using a FACSCalibur (Becton Dickinson, USA). Fig 1A shows the distribution of fluorescence of F-GMD's, and Fig 1B shows the quenching achieved by addition of anti-fluorescein Ab. Gates R1 and R2 represent fluorescent and quenched GMD's respectively. Fig 1C shows that addition of 5µl alcalase shifts the fluorescence of the GMD's into R1, thus showing that fluorescence is recovered. Fig 1D shows the FACS-analysis of a mixture of 99% quenched GMD's and 1% protease de-quenched GMD's. Approximately 0.7% of the gated GMD's are located in R1 showing that a low number of F-GMD's can be recovered.

Encapsulation of the *B. subtilis* library expressing protease was accomplished by adding around 10^6 cells (transformants) in PBS to the agarose as stated in step 4 in the protocol above. F-GMD's with cells were harvested as described above. The anti-fluorescein antibody was dialysed and 10µl Ab was added to a 1:1 mixture of GMD solution and TY containing the appropriate antibiotics to maintain the *B. subtilis* library. The GMD cultures were grown until appropriate protease expression was obtained. Alternatively the anti-fluorescein antibody can be added after an appropriate growth period, where-

after a sufficient incubation/growth period is included to allow for degradation of the antibodies by the clones producing protease.

After the growth phase the GMD cultures were harvested and resuspended in 0.2 mM Tris buffer pH7, and sorted on a FACScalibur flow cytometer (Becton Dickinson, USA). For isolation of protease active GMD's the gates were set allowing only the most fluorescent GMD's to be sorted (i.e. GMD's dequenched by action of the produced protease). GMD's were sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter. Isolated GMD's were distributed directly into microtiter plates or plated onto indicator plates for protease activity (e.g. LB plates containing 1% skim milk). Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's.

The sorted cells can be re-grown in TY medium containing appropriate antibiotics, harvested, re-embedded in F-GMD's and subjected to a second round of FACS screening as mentioned above. This screening resulted in a significant enrichment for protease positive clones.

Example 2

This example illustrates the FACS screening of a gene library for protease activity based on the biotin reloading principle in GMD's. The gene library was prepared as described in example 1.

Protease assay principle

In biotinylated agarose beads avidin is bound to all biotin molecules. Activity of a protease will remove avidin and the amount of free biotin can be measured by addition of fluorescein labeled avidin. Only GMD's containing cells with protease activity will be fluorescent.

GMD's were prepared as described in example 1 using biotinylated agarose (CelBioGel encapsulation matrix, OneCell Systems), then harvested and re-suspended in PBS.

Assay reactions were set up in 50 mM Tris pH 9 buffer by adding the appropriate amount of buffer until 50µl volumes. When 20µl Biotin-GMD's and 10µl of fluorescein conjugated Avidin (Molecular Probes; Eugene Or. Cat # A821) were mixed in a total volume of 50 µl, the beads turned fluorescent. In contrast, when F-avidin was incubated with 2µl protease (Savinase®, Novo Nordisk A/S) at 37°C in Tris pH 9.0 and 20µl of the protease treated F-avidin was added to Biotin-GMD's the fluorescence was observed to be at background levels. This shows that protease treated avidin does not bind to Biotin-GMD's, thus demonstrating that the basis for the assay is sound.

Encapsulation of a *B. subtilis* library expressing protease in Biotinylated GMD's (B-GMD's) was accomplished by adding around 10^6 cells (transformants) in PBS to the agarose as stated in step 4 in the protocol in example 1. B-GMD's with cells were harvested, and 20µl Avidin (Molecular Probes; Eugene Or. Cat # A887) was added to a 1:1 mixture of B-GMD solution and TY containing the appropriate antibiotics to maintain the *B. subtilis* library. The GMD-cultures were grown until appropriate protease expression was obtained. Alternatively the avidin can be added after an appropriate growth period, whereafter a sufficient incubation period is included to allow for degradation of avidin in the GMD's where protease is expressed.

After the growth phase the GMD cultures were harvested and re-suspended in 0.2 mM Tris buffer pH7, and 10µl F-avidin was added in order to label the biotin liberated by the action of the protease. After a second wash, the GMD's were sorted on a FACScalibur flow cytometer (Becton Dickinson, USA). For isolation of protease active GMD's, the gates were set to allow only the most fluorescent GMD's to be sorted (i.e. F-avidin is primarily bound to GMD's where unlabeled avidin is degraded by the produced protease). GMD's were sorted at a rate

corresponding to around 1000 events per second, and deposited on to a filter. Isolated GMD's was plated directly onto indicator plates for protease activity (e.g. LB plates containing 1% skim milk). Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's. The sorted cells can be re-grown in TY medium containing antibiotics, harvested, re-embedded in B-GMD's and subjected to a second round of FACS screening as mentioned above. The screening resulted in a significant enrichment for protease positive clones.

Example 3

This example illustrates the FACS screening of the gene library for protease activity based on a substrate quenching principle in GMD's.

The gene library was prepared as described in example 1.

Protease assay principle

When hemoglobin is labeled with flourescein, the fluorescence is quenched by the hemoglobin. Therefore coupling of hemoglobin to agarose via DTAF (Dichlorotriazino-5-aminofluorescein; a bi-functional fluorescein) will allow the degradation of hemoglobin by a protease to be detected as an increase in GMD associated fluorescence.

Hemoglobin de-quenching assay

Hemoglobin was labeled with DTAF by dissolving 5,66 g hemoglobin in 600 ml miliQ water. pH was adjusted to 10,0 using 4N NaOH. 95,2 mg DTAF dissolved in 4 ml DMF was added and the reaction mixture was stirred in the dark at room temperature for 24 hours. The mixture was transferred to an Amicon RA-2000 unit, equipped with a Filtron 10-kD filter, and was dialysed against miliQ water until no fluorescence could be found in the filtrate.

To show degradation of hemoglobin by protease, 100µl F-Hemoglobin at a concentration of 10 µg/ml in 50 mM bicine (Sigma) pH 9 was incubated with and without 1 µl protease (Sav-

inase®, Novo Nordisk A/S) for 60 min in 384-well microtiter plates (Nunc, Denmark). An approximate 10-fold increase in fluorescence intensity was measured on a PolarStar Galaxy (BMG LabTechnologies, Germany) in the sample with protease (Table 2). This clearly demonstrates the de-quenching of fluorescein when hemoglobin is degraded by protease.

Sample	Fluorescence intensity
1 µl savinase	40431
0 µl savinase	4749

Table 2

In order to cross-link the hemoglobin molecule to agarose using DTAF as a bi-functional linker, the agarose was first labeled with DTAF under mild conditions. Agarose was dissolved in milliQ water and the pH was adjusted to 9,0 using 4 N NaOH. DTAF dissolved in 4 ml DMF was added, and the reaction mixture was stirred in the dark at room temperature for 2-4 hours. The labeled agarose was precipitated in ETOH (96%) and washed 3 times in ETOH (96%) and once in cold water and then freeze dried. The DTAF-agarose was dissolved in milliQ water and the pH was adjusted to between 7.5-8.5, hemoglobin was added, and the reaction mixture was stirred at room temperature for up to 24 hours.

Hemoglobin-fluorescein GMD's were prepared as described in example 1.

Encapsulation of a *B. subtilis* library expressing protease in Hemoglobin-fluorescein GMD's was accomplished by adding around 10^6 cells (transformants) in PBS to the agarose as stated in step 4 in the protocol in example 1. Hemoglobin-fluorescein GMD's with cells were harvested as described in example 1.

A 1:1 mixture of Hemoglobin-fluorescein GMD containing cells and TY containing the appropriate antibiotics to maintain the *B. subtilis* library was grown until appropriate protease expression was obtained. After the growth phase the GMD cul-

tures were harvested and re-suspended in 0.2 mM Tris buffer pH7 and GMD population was sorted on a FACSCalibur flow cytometer (Becton Dickinson, USA). For isolation of protease active GMD's the gates were set to allow only the most fluorescent GMD's to be sorted (i.e. strong fluorescence is present in GMD's where protease activity has degraded the hemoglobin and the fluorescein is thus de-quenched). GMD's were sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter. Isolated GMD's were plated directly onto indicator plates for protease activity (e.g. LB plates containing 1% skim milk). Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's. The sorted cells can be re-grown in TY medium containing antibiotics, harvested, re-embedded in Hemoglobin-fluorescein GMD's and subjected to a second round of FACS screening as mentioned above. This screening resulted in a significant enrichment for protease positive clones.

Example 4

This example illustrates the FACS-screening for amylase activity based on a dye quenching or a FRET assay principle where the fluorescence in retained in GMD's. The gene library was prepared as described in example 1.

Quenching or FRET assay principle

The substrate is designed to exhibit quenching or FRET (fluorescence resonance energy transfer) in its native (non-hydrolyzed) state. Furthermore the substrate is bound to the GMD using a bi-functional fluorescent dye. Upon degradation of the substrate, the quenching or FRET is relieved, and since the fluorescent dye is bound to the agarose only GMD's containing enzyme activity will be fluorescent, or in the FRET case GMD's will fluoresce at the lower wavelength.

The substrate for this assay, an oligosaccharide (size may vary between DP 2 and DP 20) or a polysaccharide, is labeled with a fluorescent group (optionally fluorescein) and a

quenching group (optionally tetramethylrhodamine). The distance between dyes is between 10-75 Å in order for efficient FRET to occur. An oligosaccharide may be labeled with tetramethylrhodamine at the reducing end and with fluorescein at free OH groups preferably on the C6-carbon. A double functionalized fluorescent dye such as DTAF is used to couple the substrate to the agarose of the GMD, the synthesis could be performed as described is example 3.

Quenching assay based screening

In a quenching assay the substrate is designed so that fluorescein emission is quenched by a tetramethylrhodamine quencher group. When bonds in the oligosaccharide or polysaccharide are broken by enzymatic hydrolysis originating from the cells in the GMD, the fluorescein group will be de-quenched and the fluorescence will be retained in the GMD because the fluorescein also is linked to the agarose. The library will be encapsulated in GMD's as described in example 1, GMD harvested and incubated in LB or TY medium and grown until sufficient enzyme activity is present. The library will be screened by FACS, and for isolation of enzymatically active GMD's the gates will be set to allow only the most fluorescent GMD's to be sorted. GMD's will be sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter or directly into microtiter plates. Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's. The sorted cells can be re-grown in TY medium containing antibiotics, harvested, re-embedded in GMD's and subjected to a second round of FACS screening.

FRET-assay based screening

In the FRET-based assay the substrate is designed so that excitation of fluorescein will result in energy transfer to and emission from tetramethylrhodamine. When bonds in the oligosaccharide or polysaccharide are broken by enzymatic activity originating from the cells in the GMD, the tetramethylrhodamine

emission will diminish whereas the fluorescein emission will increase. The library will be encapsulated in GMD's as described in example 1, GMD's will be harvested and incubated in LB or TY medium and grown until detectable enzyme activity is present. The encapsulated library will be screened by flow cytometry. During FACS sorting the gates will be set taking changes in two colors into account; for fluorescein emission the high fluorescent population is gated and for tetramethylrhodamine emission the low fluorescent population is gated. Enzymatically active GMD's will then be sorted based on the increase in fluorescein emission or preferably on the increase in the ratio between fluorescein emission and tetramethylrhodamine emission. GMD's will be sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter or directly into microtiter plates. Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's. The sorted cells can be re-grown in TY or LB medium containing antibiotics, harvested, re-embedded in GMD's and subjected to a second round of FACS screening.

Example 5

This example illustrates the FACS-screening for amylase activity based on generating microspheres of substrate incorporated into GMD's. The gene library was prepared as described in example 1.

Hydrolase assay principle

Fluorescently labeled substrate (e.g. starch) is incorporated in GMD's by using crosslinked microspheres of such a size that they are retained in the GMD's when the substrate is intact. When substrate hydrolysis occurs the fluorescence is lost from the GMD by diffusion, and non-fluorescent GMD's are sorted.

Starch microspheres were generated as described by G. Hamdi *et al.* (G. Hamdi, G. Ponchel, and D. Duchene. An original method for studying in vitro the enzymatic degradation of

cross-linked starch microspheres. *J.Control.Release* 55 (2-3):193-201, 1998.) by epichlorohydrin crosslinking of fluorescein labeled starch (F8387, Sigma Aldrich). After crosslinking microspheres were fluorescent as observed by epifluorescence microscopy, and the diameters were between 1 to 12 μm. The average diameter of GMD's generated with the emulsification setting in example 1 is approximately 25-35 μm. Accordingly the concentration of starch microspheres was adjusted so that each GMD contained between 1 and 5 microspheres. To incorporate the starch microspheres in the GMD's, the GMD's are generated as described in example 1 except that in step 2 also 30 μl microspheres are added to the molten agarose and in step 4 only 70μl cells are added. GMD's containing microspheres and cells are harvested and washed as described in example 1.

The library will be encapsulated in GMD's containing labeled starch microspheres as described above, GMD's can be harvested and incubated in LB or TY medium containing appropriate antibiotics and grown until detectable enzyme activity is present. The encapsulated library can be screened by flow cytometry.

During FACS sorting the gates can be set to select the fraction of GMD's which have lost their fluorescence (i.e. the least fluorescent population is sorted). Since positive clones are expected to lose fluorescence it could be advantageous to include a cell stain in the assay in order to stain GMD's with microcolonies. The cell stain should emit at a wavelength different than fluorescein, and could be a membrane probe such as the red fluorescent DiI (Molecular Probes). Gates can in this case be set to identify GMD's with microcolonies (red fluorescent) which have lost their green fluorescence, indicative of breakdown and leakage of fluorescein labeled starch. GMD's can be sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter or directly into microtiter plates. Optionally agarase can be added in order to aid outgrowth of the sorted clones from the GMD's. The sorted cells can be re-grown in TY or LB medium containing antibiotics, har-

vested, re-embedded in GMD's and subjected to a second round of FACS screening.

Example 6

This example describes a growth method that allows screening of filamentous fungi in GMD's. It is a difficult task to make libraries in filamentous fungi. One of the problems is the different morphology and growth rate between different clones that have been transformed with a DNA library of interest. This gives rise to large and small clones, as well as clones that may be outcompeted by other clones. Furthermore hypha will grow out of the GMD's making FACS sorting impossible. Assaying fungal transformants of different sizes is also a difficult task due to differences in amounts of expressed enzymes.

These problems can be overcome by growing the clones individually in agarose GMDs with a high molecular weight (HMW) dextran as carbon source. This growth mode provides:

1) uniform colony size (comparable level of enzyme expressed)
2) no cross-contamination between clones (slow growing clones are allowed to grow out without contamination from fast growing clones)
3) prevents that hypha grows out of the GMD

Growth in GMD's with HMW dextrane as C-source

Transformation was performed as described in WO 98/01470, and 10% of the transformed protoplasts were plated and cultured on Cove plates at 37°C as a control, showing that the procedure does not harm the transformation frequency.

The protoplasts should be resuspended in the agarose solution (4% agarose, 2% high molecular weight dextran ($5-40*10^6$ kd), 1.2 M sorbitol, 10 mM TRIS pH7.5), and encapsulated as described in example 1.

The protoplast containing GMD's should be grown for a desired period of time at 30°C degrees in STC (10 mM CaCl2, 1.2 M sorbitol, 10 mM TRIS pH7.5) in order to regenerate the cell wall. After a couple of washes in sterile water to remove sor-

bitol the GMD's are transferred to 1* Vogel media and grown at 30°C degrees for any desired time period, either to express the enzyme of interest and/or to get a reasonable biomass. The sole carbon-source is the dextran in the GMD's. This prevents cross-contamination from GMD to GMD, and allows slow growing transformants to gain reasonable biomass, as well as confining growth to the volume of the GMD. The GMD's with live transformants can be screened using a FACS-based assay as described in examples 1-5 and 7.

Example 7

This example describes a FACS screening method for enrichment of fungal cells secreting a material with antimicrobial activity. Although the current example describes the identification of antifungal protein (AFP) variants with improved properties, e.g. species specificity or potency, the described method can also be employed to identify cells secreting novel antimicrobials.

AFP from *Aspergillus giganteus* has a broad and potent anti-fungal activity and a limited but significant activity against both Gram-positive and Gram-negative bacteria. AFP is a small protein of only 51 amino acids, and it is extremely heat, pH- and protease stable. Using directed evolution in combination with a FACS screening system, the antimicrobial activity towards fungi, yeast and bacteria can be optimized.

Mutant libraries of the AFP gene are generated by methods known in the art; for instance, random mutagenesis as disclosed in US-patent 4,894,331 and patent-application WO 93/01285; or shuffling of existing mutants as described in WO 95/22625. The mutant libraries are established in the filamentous fungus *Aspergillus oryzae* by protoplast transformation as described in WO 98/01470.

Transformed protoplasts or spores are encapsulated as described in example 1. The encapsulation is done in such a way that most beads are clonal, e.g. initially is seeded with one cell expressing only one variant.

GMD's were harvested and encapsulated protoplasts were regenerated and grown until sufficient biomass had developed using the method described in example 6.

An AFP-sensitive tester strain can be included. If the tester strain grows slower than *Aspergillus oryzae*, both organisms can be included in the GMD simultaneously. If, however, the tester strain grows faster than the producing organism, a secondary agarose encapsulation providing the tester strain will have to be performed. These double agarose gel microdrops are simply GMD's within GMD's, and can be prepared as described in Gift *et al.*, 1996 (Nature Biotechnology, Vol. 14, 884-887). The concentration of the tester strain, initially *Shewanella putrefaciens*, can be adjusted to a 10-1000 fold excess over the biomass in the GMD's containing the library of AFP. After a sufficient incubation time where the AFP has been produced by the fungal clone and allowed to act on the tester strain the GMD's will be stained with Live/Dead BacLight viability stains (see below for references) for about 10 min and subjected to FACS sorting. During FACS sorting the gates will be set to select the fraction of GMD's which have increased red fluorescence (indicative of dead tester cells) and which have simultaneously lost their green fluorescence (indicative of live tester cells). GMD's can be sorted at a rate corresponding to around 1000 events per second, and deposited on to a filter or directly into microtiter plates. Upon identification, the interesting fungal clones can be grown individually and analyzed with respect to AFP potency, species specificity, etc. using more traditional methods.

<u>References on live/dead or viability stains:</u>

Molecular probes catalogue 15.2 (http://www.probes.com/handbook/sections/1502.html). The procedures for using such probes are available from the manufacturer. Also fluorescent redox probes, sensitive towards the cross membrane potential, may be employed (Rodriguez, et al., 1992, Use of a Fluorescent Redox Probe for Direct Visualization of Actively Respiring Bac-

teria, Appl. Environ. Microbiol., pp. 1801-1808) wherein a 5-cuano-2,3-ditolyltetrazolium chloride probe is described. (Jepras R.I., et al., 1997, Rapid Assessment of Antibiotic Effects on Escherichia coli by bis-(1,3-Dibutylbarbituric Acid) trimethine Oxonol and Flow cytometry, Antimicrobial Agents and Chemotherapy; pp. 2001-2005) wherein a DiBAC4(3) probe available from Molecular Probes is employed.

CLAIMS

1. A method for screening a DNA library for DNA of interest comprising the steps of:

a) creating host cells comprising the DNA library,

b) generating samples each comprising a host cell of step a),

c) establishing a means for correlating host cell secretion of a material of interest in a sample with the fluorescence of the sample,

d) determining which intensity interval of fluorescence indicates secretion in the sample when the correlating means of step c) is used,

e) cultivating the samples under suitable conditions, and

f) selecting the samples exhibiting fluorescence within the intensity interval of step d) using a fluorescence analyzer; wherein the host cell comprises DNA of interest.

2. The method according to claim 1, wherein the DNA library is generated from a natural DNA sequence by DNA shuffling or directed evolution.

3. The method according to claim 1 or 2, wherein the host cells of claim 1 step a) are of bacterial or fungal origin.

4. The method according to claim 3, wherein the host cells of claim 1 step a) are *Escherichia coli*, *Bacillus subtilis* or *Aspergillus oryzae*.

5. The method according to any of claims 1-4, wherein the samples of claim 1 step b) are micro-environments.

6. The method according to claim 5, wherein the micro-environments are liposomes, beads, cells, ghost red blood cells, or ghost macrophages.

7. The method according to claim 5, wherein the micro-environments are gel micro drops (GMD's) comprising agarose, polysaccharide, carbohydrate, alginate, carrageenan, chitosan, cellulose, pectin, dextran or polyacrylamide.

8. The method according to claim 7, wherein the GMD's comprise as the essentially sole carbon source high molecular weight (HMW) dextran.

9. The method according to any of claims 5-8, wherein the micro-environments are encapsulated by a layer, said layer being essentially non-penetrable to the material of interest, preferably the layer comprises a lipid material.

10. The method according to any of claims 1-9, wherein the material of interest of claim 1 step c) is a polypeptide.

11. The method according to claim 10, wherein the polypeptide is an enzyme selected from the group consisting of lipases, esterases, proteases, glucosidases, glucosyl transferases, phosphatases, phytases, kinases, mono- and dioxygenases, haloperoxidases, peroxidases, transaminases, rhamnogalacturonases, lactases, and laccases.

12. The method according to claim 10, wherein the polypeptide is an antimicrobial agent, a growth-promoting agent, or a pharmaceutical peptide.

13. The method according to any of claims 1-9, wherein the material of interest of claim 1 step c) is a pharmaceutical compound.

14. The method according to any of claims 1-13, wherein the means of claim 1 step c) is based on labelling the sample with

a fluorescent group, preferably fluorescein, more preferably dichlorotriazino-5-amino-fluorescein (DTAF).

15. The method according to claim 14, wherein the means of claim 1 step c) is further based on Fluorescence Resonance Energy Transfer (FRET), quenching of the fluorescent group, or de-quenching of the fluorescent group.

16. The method according to claim 15, wherein the FRET, quenching or de-quenching is achieved by using a polypeptide, preferably rhodamine, more preferably hemoglobin, even more preferably casein, and most preferably antibodies directed towards the fluorescent group.

17. The method according to any of claims 1-13, wherein the material of interest is a protease, and wherein the means of claim 1 step c) is based on labeling the sample with a bi-functional fluorescent dye, and where the fluorescence of said dye is quenched by anti-fluorescein antibodies, or by a protein covalently linked to the dye, preferably the bi-functional dye is dichlorotriazino-5-amino-fluorescein (DTAF).

18. The method according to any of claims 1-13, wherein the material of interest is a protease, and wherein the means of claim 1 step c) is based on labeling the sample with biotin and conjugating the biotin with fluorescently labeled avidin or streptavidin.

19. The method according to any of claims 1-13, wherein the material of interest is an amylase, and wherein the means of claim 1 step c) is based on quenching/de-quenching or FRET in a substrate construct having the following structure: agarose-(bi-functional fluorescent dye)-substrate-rhodamine, where the substrate is at least in part degraded by the amylase thereby effectively releasing the rhodamine.

20. The method according to any of claims 1-13, wherein the means of claim 1 step c) is based on a fluorescently labeled degradable substrate which when present in the samples of claim 1 step b) is non-diffusable in its un-degraded form and diffusable when degraded, preferably the substrate is fluorescently labeled substrate microspheres, preferably starch microspheres.

21. The method according to any of claims 1-13, wherein the material of interest is an anti-microbial peptide, and where the samples of claim 1 step b) further comprise a tester cell that displays a detectable response to the anti-microbial peptide.

22. The method according to any of claims 1-21, wherein the fluorescence analyzer of claim 1 step f) is a Fluorescence Activated Cell Sorter (FACS).

23. A recombinant vector comprising DNA isolated by a method as defined in any of claims 1-22.

24. A recombinant host cell comprising DNA isolated by a method as described in any of claims 1-22 or the vector according to claim 23.

25. The cell according to claim 24, which is of microbial origin.

26. The cell according to claim 25, which is of *Escherichia*, *Bacillus* or an *Aspergillus* origin.

27. The cell according to claim 26, which is chosen from the group consisting of: *Eschericia coli*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus clausii*, *Aspergillus niger*, *Aspergillus oryzae*, and *Aspergillus nidulans*.

28. A transgenic animal containing and expressing DNA isolated by a method as defined in any of claims 1-22.

29. A transgenic plant containing and expressing DNA isolated by a method as defined in any of claims 1-22.

30. A method of producing a material of interest, which method comprises cultivating a cell according to any of claims 24-27 in suitable culture medium under conditions permitting expression of the DNA of interest and recovering the resulting material from the culture medium.

31. A method of producing a material of interest, which method comprises recovering the material from any part or secrete of/from the transgenic animal according to claim 28.

32. A method of producing a material of interest, which method comprises growing a cell of a transgenic plant according to claim 29, and recovering the material from the resulting plant.

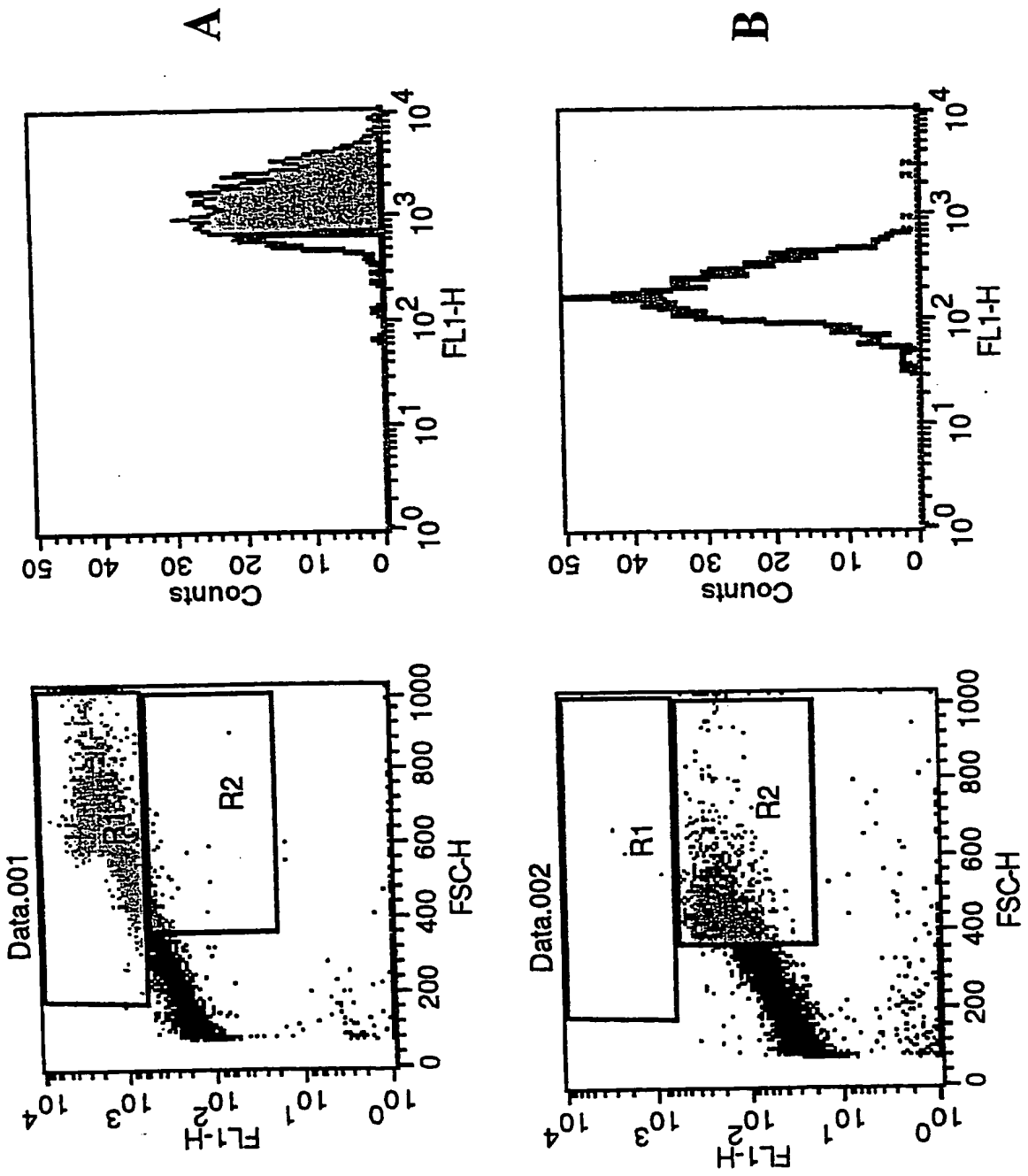

Fig. 1

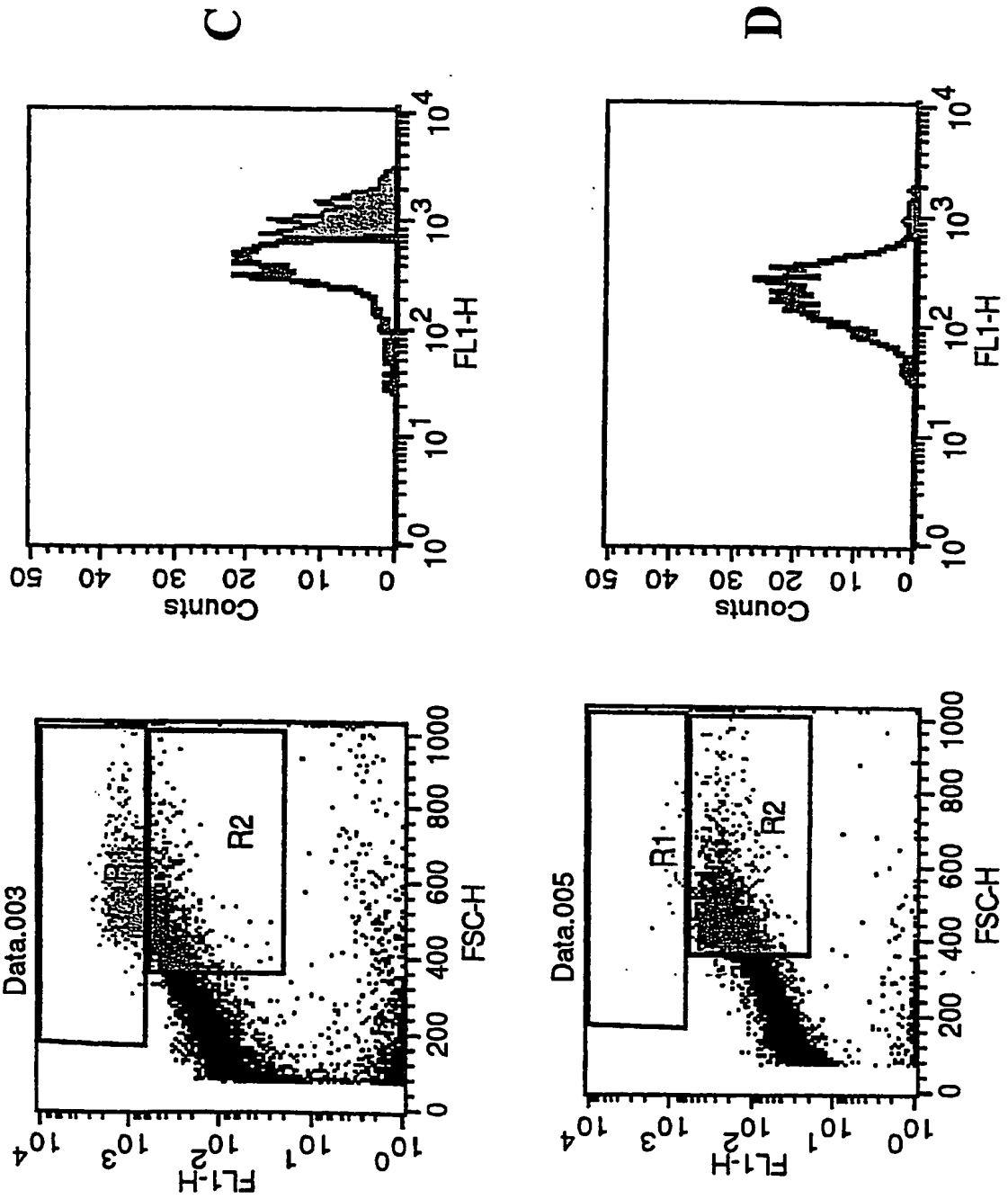

Fig. 1 (continued)